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Research paper

Development of a sensitive method to extract and detect low numbers of *Cryptosporidium* oocysts from adult cattle faecal samples

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ABSTRACT

Cryptosporidium transmission studies to date have concluded that adult cattle are not a significant source of oocysts contributing to clinical cryptosporidiosis in calves on farm. However current methods of sample processing have been optimised for calf faecal samples and may be less sensitive when used on adult samples due to lower numbers of oocysts and larger size of samples. A modified and novel method of oocyst extraction and concentration was developed and applied in an experiment involving spiking adult cattle faecal samples with known concentrations of *Cryptosporidium* oocysts. The results showed an increased sensitivity of detection from 100 oocysts/g of faecal sample using conventional protocols to 5 oocysts/g using the newly developed method. As it is important to be able to accurately assess the contribution of adult ruminants to the transmission of *Cryptosporidium*, both on farm and in the environment, the development of the techniques described here is likely to make an important contribution to *Cryptosporidium* transmission studies in future and in subsequent control strategies aimed at the reduction of *Cryptosporidium* infection in calves on farm.

1. Introduction

The protozoan parasite *Cryptosporidium* is well documented as a major cause of neonatal enteritis in farm livestock particularly calves (Silverlas and Blanco-Penedo, 2013; Santin, 2013; Rieux et al., 2013; Chalmers and Katzer, 2013). Detection of the parasite in calf faeces is straightforward due to the high number of oocysts and small samples and is achieved either by microscopy or direct DNA extraction from faecal material following lysis of the oocysts. In comparison, parasite detection in adult cattle presents a problem due to the large samples, lower concentration of oocysts and the fibrous nature of the faeces.

Some studies investigating *Cryptosporidium* in adult cattle faeces have concluded that adult cattle are not a significant source of the parasite either in terms of transmission to calves or contamination of the environment (Atwill and Pereira, 2003; DE Waele et al., 2012). It is possible that sub-clinical/asymptomatic infections of older animals are simply being missed because current methods for detection of *Cryptosporidium* in adult cattle faeces are not sensitive enough. Many of these studies have used only a small sub-sample of faecal material from 1 to 20 g and either a centrifugation or flotation method to concentrate the oocysts (Silverlas and Blanco-Penedo, 2013; Ralston et al., 2010; Fayer et al., 2000; Atwill and Pereira, 2003; Smith et al., 2014; Wang et al., 2011). In addition, these studies, even the most recent, have relied on various microscopy techniques for identification of positive samples (Silverlas and Blanco-Penedo, 2013; Smith et al., 2014). Although there is no 'gold-standard' for detection of *Cryptosporidium* oocysts in faeces it is generally accepted that microscopy is less sensitive than PCR methods (Chalmers et al., 2011) and again low-level shedding by adult cattle may have been missed and no speciation is possible.

Here we describe a method which increases detection of oocysts in adult cattle samples by using a large starting sample and performing a concentration and flotation step prior to freeze thawing the oocysts in liquid nitrogen for 10 cycles, followed by DNA extraction and nested PCR. Due to the length of the process involved, it is anticipated that this method would mainly be applicable to research laboratories but may have a large impact on interpretation of parasite transmission and thereby on control strategies advised for parasite reduction on farm and in the environment.

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Table 1
Experimental design for spiking experiment to establish the comparative sensitivities of the acid flocculation method of oocyst concentration compared to other methods. AF = acid flocculation; SF = salt flotation; Direct = DNA extraction direct from sample.

	Method			
No. oocysts per gram	Direct	AF	AF and SF	SF
0	Loop −250 mg	50 g	50 g	3 g
5	Loop −250 mg	50 g	50 g	3 g
10	Loop −250 mg	50 g	50 g	3 g
100	Loop −250 mg	50 g	50 g	3 g
1000	Loop −250 mg	50 g	50 g	3 g

2. Methods

2.1. Spiking experiment

Faecal samples were collected (from the floor) from a yearling bull housed at Glasgow University Veterinary School since birth, therefore more likely to be free of *Cryptosporidium* oocysts. The samples were pooled and mixed thoroughly prior to processing; DNA extracted and a nested species specific multiplex-PCR (nssm-PCR) performed (Thomson et al., 2016). The sample was then split for the different processing methods (acid flocculation only; acid flocculation and salt flotation; salt flotation only; direct DNA extraction) to be compared and then spiked by mixing the faeces with the relevant oocyst numbers as shown in Table 1. Each processing method sample was further split into five to apply the different oocyst concentrations (0; 5; 10; 100; and 1000 oocysts/g). The oocysts used for spiking came from the same dilution series and were obtained from calves (n = 2) experimentally infected with *C. parvum* at the Moredun Research Institute as part of another study. The same oocyst preparations were used for each processing method. Tests were conducted from the same samples on two plates. Each combination of processing method and concentration were run in triplicate on both plates. Experimentally infected animals were used in accordance with Home Office regulations approval from the Moredun Research Institute's Ethical Review Committee.

2.2. Acid flocculation

The acid flocculation method used here was based on the technique described by Ortega-Mora and Wright (1994). Spiked faecal samples were well mixed and 50 g added to a 1 l cylinder with 600 ml water and 7 ml 0.37 M sulphuric acid (H_2SO_4). The sample was put on a magnetic stirrer for 5 min then left to settle for approximately 15 min or until a clear line was visible between the sediment and supernatant, after which the supernatant was removed by pipette, collected and centrifuged for 20 min at 1000 × *g*. The supernatant was discarded and 6 ml H_20 was added to the pellet, mixed and put into a 15 ml tube, which was centrifuged for 5 min at 3000 × *g*. The supernatant was discarded and the pellet retained for further processing either DNA extraction or salt flotation. The extra centrifugation and resuspension step included in this method was necessary to remove as much of the fibrous material associated with adult cattle faecal samples as possible, for successful DNA extraction using extraction columns.

2.3. Salt flotation

A 3 g faecal sample or pellet from acid flocculation was added to 8 ml saturated salt solution and thoroughly mixed by vortexing and 2 ml dH_2O trickled on top of the salt solution before centrifugation at 1000 × *g* for 8 min. Following centrifugation the water layer was gently swirled using a Pasteur pipette to create a vortex drawing the oocysts from the layer between the dH_2O and salt into the dH_2O. This layer containing the oocysts was removed and added to 6 ml dH_2O. The total volume was made up to 10 ml; the sample was mixed by inverting and then centrifuged at 5000 × *g* for 5 min (Elwin et al., 2001; Ryley et al., 1976). The supernatant was poured off and discarded and the pellet retained for DNA extraction.

2.4. DNA extraction using a modified macherary-nagel tissue kit protocol

Prior to DNA extraction, either one loop (250 mg) of faecal material taken directly from faeces, or the pellet obtained following salt flotation, acid flocculation or both was resuspended in 1 ml TE buffer(10 mM Tris-HCl, 0.5 mM EDTA) mixed vigorously and centrifuged at 5000 × *g* for 10 mins. The pellet was then resuspended in 200 μl lysis buffer (T1 buffer, Macherey-Nagel, NZ740952250) and 10 freeze-thaw cycles in liquid nitrogen to disrupt the oocyst wall and a water bath at 56 °C were performed. DNA was extracted using NucleoSpin Tissue DNA, RNA and Protein Purification Kits (Macherey-Nagel, NZ740952250) following the manufacturer's protocol with the following modifications: the samples were incubated with Proteinase K at 56 °C overnight following which the samples were vortexed vigorously. Prior to the addition of ethanol, the samples were centrifuged at 11,000 × *g* for 5 mins to remove insoluble particles and the supernatant retained. Ultrapure water (100 μl) was used to elute DNA which was then stored at −20 °C until required.

2.5. Polymerase chain reaction

DNA was amplified using the nssm-PCR described by (Thomson et al., 2016). Briefly each 25 μl reaction contained 10× PCR buffer (45 mM Tris-HCl pH 8.8, 11 mM (NH4)2SO4, 4.5 mM MgCl2, 4.4 μM EDTA, 113 μg/ml BSA, 1 mM each of the four deoxyribonucleotide triphosphates) (MacLeod et al., 1999), 0.5 units BioTaq (Bioline, UK), 10 μM of each primer (Thomson et al., 2016) and 3 μl DNA in the primary round and 1 μl primary PCR product in the secondary round. The total volume was made up to 25 μl with dH_2O. In each PCR run one set of positive controls, DNA extraction and negative controls consisting of dH_2O were included. All reactions were carried out in triplicate. Cycling conditions were 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C and 1 min at 72 °C. The final extension was 7 min at 72 °C. Secondary amplification products (~3 μl) were visualised following electrophoresis on a 1.5% agarose gel stained with GelRed™ (Biotium, UK) on an AlphaImager 2000.

2.6. Statistical analysis

Detection of *Cryptosporidium* parasites (positive or negative) by the different processing methods was analysed as binary using logistic regression, incorporating a penalized likelihood approach (Firth, 1993) to deal with the issue of sparse data (that is, lack of variation in the binary measure). The variables modelled were whether the samples had been spiked with oocysts or not, oocyst concentration (0;5;10;100 and 1000 oocysts/g) and processing method applied (AF&SF; AF; SF and DR). Since the faeces were homogenised it was assumed that there is negligible variability between the four samples taken to which the methods were subsequently applied and since the same oocyst preparations of each concentration were used for each method it was also assumed that there is negligible variability between the samples spiked with each oocyst concentration. Proportions of positive replicates on the two plates were identical and so no plate effect was included in the model. Statis-

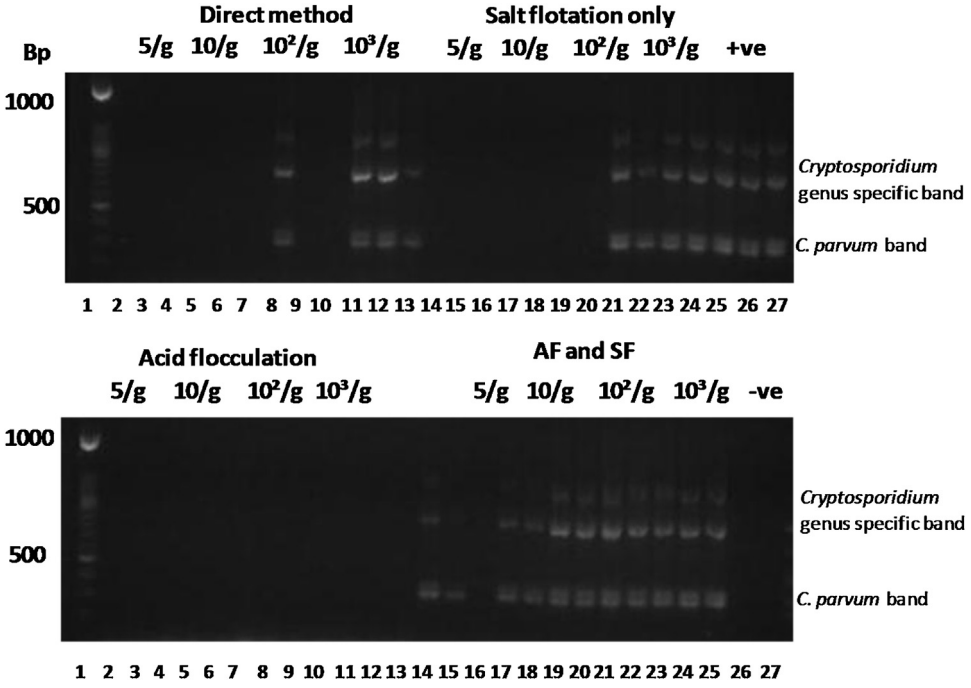

Fig. 1. Gel image of the spiking experiment comparing direct, AF, SF and AF&SF methods of oocyst extraction. PCR reactions were run in triplicate.
Top image: lane 1 = DNA Molecular Weight Marker X1V 100 base pair ladder(Roche, 11 721 933 001); lanes 2–13 = direct method: 2–4 = 5 oocysts/g; 5–7 = 10 oocysts/g; 8–10 = 10^2 oocysts/g; 11–13 = 10^3 oocysts/g. Lanes 14–25 = Salt flotation only: 14–16 = 5 oocysts/g; 17–19 = 10 oocysts/g; 20–22 = 10^2 oocysts/g; 23–25 = 10^3 oocysts/g. Lanes 26–27 = positive controls.
Bottom image: lane 1 = marker (Roche 100 bp); lanes 2–13 = acid flocculation only: 2–4 = 5 oocysts/g; 5–7 = 10 oocysts/g; 8–10 = 10^2 oocysts/g; 11–13 = 10^3 oocysts/g. Lanes 14–25 = Acid flocculation and salt flotation: 14–16 = 5 oocysts/g; 17–19 = 10 oocysts/g; 20–22 = 10^2 oocysts/g; 23–25 = 10^3 oocysts/g. Lanes 26–27 = negative controls.

Table 2
Results of the spiking experiment comparing direct, AF, SF and AF&SF methods of oocyst extraction. All PCR reactions were run in triplicate. Plate two results were identical.

No. Oocysts per gram	Direct method	AF only	SF only	AF and SF
0	Negative	Negative	Negative	Negative
5	Negative	Negative	Negative	2/3 positive
10	Negative	Negative	Negative	3/3 positive
100	1/3 positive	Negative	2/3 positive	3/3 positive
1000	3/3 positive	Negative	3/3 positive	3/3 positive

tical analysis was conducted in R version 3.3.0 using the function logistf.

3. Results

3.1. Spiking experiment

The results of the spiking experiment comparing the 4 methods of oocyst extraction from adult cattle faeces are given in Table 2 and Fig. 1. The direct method was sensitive to 100 oocysts/g of faeces; AF alone failed to allow detection of oocysts; SF alone was sensitive to 100 oocysts/g and the combined AF&SF method to 5 oocysts/g.

3.2. Statistical analysis

The results from the logistic regression showed that overall AF followed by SF (AF&SF) is much better at identifying the presence of *Cryptosporidium* (main effects of processing method: AF&SF vs DR P-value < 0.001; AF&SF vs SF P-value < 0.001; and AF&SF vs AF P-value < 0.001). The results also indicated that AF&SF is more sensitive than the other methods since it detected the parasite in samples with only 5 oocysts per gram of faeces compared to the other methods where the lowest detected concentration was 100 oocysts per gram. The interactions between oocyst concentration and method were not statistically significant (interactions of oocyst concentration and processing method: AF&SF vs DR P-value = 0.172; AF&SF vs AF P-value = 0.957; and AF&SF vs SF P-value = 0.207).

4. Discussion

Previous reports on the prevalence of *Cryptosporidium* in adult cattle have shown large variations ranging from 0 to 71% (Lorenzo Lorenzo et al., 1993; Atwill and Pereira, 2003) although the majority have reported prevalence of less than 7% and concluded that adult cattle do not make a significant contribution to *Cryptosporidium* burden in calves or in the environment (DE Waele et al., 2012; Atwill and Pereira, 2003). The variation in results may be due to the small weight of faecal material used for analysis and the techniques used to concentrate and extract the oocysts from the adult faeces. In most studies from 1 to 20 g of faeces has been used as starting material, followed by salt or sucrose flotation as a method of oocyst extraction (Silverlas and Blanco-Penedo, 2013; Wang et al., 2011; Ralston et al., 2010; Faubert and Litvinsky, 2000; Fayer et al., 2007).

In comparison to calves, adult cattle faecal samples are very large, highly fibrous and have relatively low amounts of *Cryptosporidium* oocysts present. The new techniques used in this study were developed to enable detection of low numbers of *Cryptosporidium* oocysts in faeces collected from adult cattle. For example, using AF followed by SF, enabled detection down

to 5 oocysts/g by 18S nssm-PCR in this study, compared to 100 oocysts/g when SF only was used (Table 2). The increased sensitivity achieved using AF and SF is probably due to a combination of the larger starting sample (50 g) which is possible due to the AF method separating the oocysts from most of the fibrous faecal material. When this method is followed with SF there is very little debris left in the final pellet and as oocysts have a tendency to adhere to fibrous material, the cleaner samples potentially allow for more effective downstream analysis, specifically the freeze thawing to disrupt oocyst walls and DNA extraction using mini columns. The failure of detection using AF only in this study was due to attempting to use the whole pellet obtained from AF of 50 g of faecal material, which proved too large to enable the successful extraction of DNA as the remaining fibre blocked the DNA extraction columns. In conclusion, the methods developed in this study using AF followed by SF, resulted in detection of *Cryptosporidium* oocysts in spiked samples containing 5 oocysts/g compared to the next most sensitive method, SF only, which resulted in detection at 100 oocysts/g. The results obtained from this optimised method of concentrating oocysts has already had important consequences for the estimation of *Cryptosporidium* prevalence in farmed livestock and wildlife (Wells et al., 2015) and may lead to a different interpretation of transmission of the parasite as it is applied to further field studies. It is crucial to be able to accurately assess the contribution of adult ruminants to the transmission of *Cryptosporidium* as the adults are a constant presence and, even if they carry the parasite in low numbers, are likely to make a large contribution to parasite transmission and prevalence. The use of the techniques described here is therefore likely to make an important contribution to *Cryptosporidium* transmission studies in future.

Conflict of interests

All authors declare that they have no competing interests.

Author's contribution

BW, ST and FK designed the study. BW and ST performed the spiking experiment. BW, ST, FK and LI drafted the manuscript. HE performed the statistical analysis.

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